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The Effect of Hypoxia on G Protein Coupled (CB_1) Receptor Gene Expression in Cortical B50 Neurons in Culture

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Abstract: Hypoxia adversely affects cells and tissues, and neuronal cells in particular have been shown to be more susceptible to the injurious effects of hypoxia in which they may begin to die when oxygen supply is reduced or completely eliminated. Cannabinoid (CB_1) receptor agonists have been shown to elicit several Central Nervous System (CNS) effects, mediated via G protein-coupled receptors. The aim of this study was to examine the effect of hypoxia on G protein coupled receptor (CB_1) gene expression in cortical neuronal B50 cell lines in culture. The B50 cells were cultured in normoxia (21% O_2; 5% CO_2) and hypoxia (5% O_2; 5% CO_2), and were treated with cannabinoid agonists to determine their effects on hypoxia-induced changes. Three cannabinoid agonists [Win55,212-2 mesylate (Win), arachidonoylethanolamide (AEA) and 2-arachidonylglycerol (2-AG)], were administered to the cells as treatment for 48 hours after 48hours of initial culture for a total of 96hours of culture in hypoxic conditions at concentrations of 10, 50 and 100 nM. The levels of G-protein coupled receptor (CB1) mRNAs were assessed using RT-PCR. The results showed that hypoxia induced morphological changes in B50 cells in hypoxia while the CB_1 RT-PCR mRNA levels showed no appreciable changes in normal, hypoxic and treated cells. The results show that B50 neuronal cells are susceptible to damage and injurious effects of hypoxia, as are most brain cells and the cannabinoid agonist treatments showed there were no changes in the level of CB_1 receptor gene expression due to hypoxia or agonist treatment in neuronal B50 cells in culture.

Key words: Cannabinoid (CB_1) receptor, cannabinoid agonist, G-protein coupled receptor, hypoxia, messenger RNA, reverse transcription-PCR

INTRODUCTION

Cannabinoid (CB_1) receptors are concentrated mainly in the cerebellum and the basal ganglia, as well as in the hippocampus and the amygdala (Gardner, 2006; Begg *et al*., 2005). CB_1 receptors are predominantly found in the neurons of the Central Nervous System (CNS) and the B50 neuronal cells are derived from the CNS neurons (Matsuda *et al*., 1990; Soderstrom and Johnson, 2000). A second cannabinoid receptor was initially detected in spleen cells, white blood cells, and other tissues associated with the immune system. This second receptor is called the CB_2 receptor and is mainly present in the peripheral system (Munro *et al*., 1993). William Devane and colleagues were the first to identify the original endogenous cannabinoid called arachidonyl ethanolamine (AEA), or "anandamide" (Devane *et al*., 1992). The AEA is a brain-derived lipid that binds to cannabinoid receptors and mimics the biological effects of Δ^9-THC (Begg *et al*., 2005). Some years later, the second endocannabinoid was isolated from the intestinal tract and brain, called 2-arachidonoylglycerol (2-AG) (Sugiura *et al*., 1995).

The biological effects of endogenous, plant-derived and synthetic cannabinoids are mediated through specific G protein coupled cannabinoid (CB) receptors. The CB_1 receptor is highly conserved in mice, rats and humans while the CB_2 receptors are more divergent (Begg *et al*., 2005). Both CB_1 and CB_2 receptors are coupled through $G_{i/o}$ proteins to inhibit adenylyl cyclase and regulate calcium (↓) and potassium (↑) channels (Begg *et al*., 2005; Mackie, 2006). In tissues naturally expressing CB receptors and in transfected cell lines, CB_1 and CB_2 receptors have been shown to have a high level of ligand-independent activation (Begg *et al*., 2005). It has been shown that in the population of wild-type CB_1 receptors, only about 30% exists in the activated form while 70% are inactive (Kearn *et al*., 1999; Carter and

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Weydt, 2002). Some of the CB_1 receptors exist in inactivated form within the cytosol and are in GDP-bound state while some exist in a tonically activated state and are coupled to active G-proteins within the plasma membrane in their GTP-bound state (Vásquez and Lewis, 1999; Nie and Lewis, 2001). It has been shown that in their activated state, the receptors have a higher affinity for the cannabinoid agonists (Vásquez and Lewis, 1999; Nie and Lewis, 2001). The human CB_2 receptors show 68% amino acid homology with the CB_1 receptors in the transmembrane domains and a 44% overall homology (Munro *et al*., 1993; Begg *et al*., 2005). However, despite the low level of homology between the two receptors, their pharmacology is similar with most plant-derived, endogenous and classical synthetic cannabinoids having similar affinities for the two receptors (Showalter *et al*., 2005; Begg *et al*., 2005), although synthetic agonists with greater than 100-fold affinity for CB_1 or CB_2 receptors have been developed (Hillard *et al*., 1997; Malan *et al*., 2001). Both CB_1 and CB_2 receptors are the primary targets of endogenous cannabinoids and they play important role in many processes, including metabolic regulation, craving, pain, anxiety, bone growth, and immune function (Mackie, 2006). There is evidence of agonist selectivity for CB_1 receptors coupled to different subtypes of G_i proteins or to G_i versus G_o proteins (Howlett, 2004). Reverse Transcription (RT) Polymerase Chain Reaction (PCR), is a laboratory method used for amplifying a piece of ribonucleic acid (RNA) molecule (Prasad *et al*., 2001). The RNA strand is first reverse-transcribed into its deoxyribonucleic acid (DNA) complement or complementary DNA, followed by amplification of the resulting DNA using a polymerase chain reaction (Prasad *et al*., 2001). The RT-PCR based assay is one of the most common methods for characterizing and confirming gene expression patterns using messenger ribonucleic acid (mRNA) in different sample populations (Orlando *et al*., 1998; Bustin, 2002). The amplification of RNA using RT-PCR provides for a highly sensitive technique, where a very low copy number of RNA molecules can be detected and is widely used in the diagnosis of genetic diseases and, as a measure of gene expression (Nolan *et al*., 2006; Burdyga *et al*., 2004). Accurate determination of total RNA concentration is important for the quantification of mRNA levels. The RT is important for sensitive and accurate quantification and the amount of cDNA produced by the reverse transcriptase accurately represents the RNA input (Gnanapavan *et al*., 2002; Wall and Edwards, 2002; Liu *et al*., 2002). The RT-PCR quantification of mRNA has been used to monitor transcription *in vitro* and direct detection of the effects of receptor signalling (Liu *et al*., 2002; Yuen *et al*., 2002; Cohen *et al*., 2002). The RT-PCR method has also been used to study the effects of some experimental agents on the expression of cannabinoid receptors in whole animal and culture conditions (Lalonde *et al*., 2006; Chen *et al*., 2005; Zhang *et al*., 2006). The aims of the present work were to investigate the expression of cannabinoid (CB_1) receptor gene in B50 neuronal cells. To investigate the effect of hypoxia on the expression of cannabinoid (CB_1) receptor gene on B50 neuronal cells and to investigate the effect of cannabinoid receptor agonist treatment on cannabinoid (CB_1) receptor gene in cultured B50 cells using semi quantitative RT-PCR.

MATERIALS AND METHODS

Neuronal culture: One group of B50 neuronal cells were cultured and maintained in a normoxic incubator (21%O_2; 5% CO_2) as control group and another batch cultured under hypoxia (5% O_2; 5% CO_2) as experimental group. Cells were cultured in 12-well culture plates for 48 hours and three cannabinoid receptor agonists: Win55, 212-2 mesylate (Win), anandamide or arachidonoylethanolamide (AEA), and 2-arachidonylglycerol (2-AG), and antagonists were administered to the cells as treatment against hypoxia for 48 hours for a total of 96 hours at a concentration of 10, 50 and 100 nM. The total cellular RNA was extracted from the cultured B50 neuronal cells using the TRIzol reagent method (Invitrogen No 15596-026), as outlined below. The cells were cultured at the cell culture Laboratory of Queen Margaret University Edinburgh and the RT-PCR was done at the Centre for Neuroscience, University of Edinburgh United Kingdom in 2006.

Method: The B50 cells in different experimental groups were grown and lysed in culture plates by adding 0.5ml of TRIzol reagent to each well. The cells were homogenized and incubated for 5 min at room temperature. The homogenates were transferred to micro-centrifuge tubes, 0.1 mL of chloroform added, the cap secured and the tubes shaken vigorously by hand for 15 sec. The cellular mixture was incubated at room temperature for 3 min. The mixture was then centrifuged at 12,000 x g for 15 min at room temperature. Following centrifugation, the mixture, separated into 3-layers namely a lower phenol-chloroform phase (Red), a middle interphase (Cloudy) and an upper aqueous colourless phase.

The RNA was present at the upper aqueous colourless phase and formed about 60% of the total volume of the mixture and was transferred to a fresh micro-centrifuge tube. The aqueous phase was mixed with 0.25 mL of isopropyl alcohol and incubated for 10 min at room temperature. The mixture was then centrifuged at 12,000 x g for 10 min at room temperature. At this point the RNA precipitated and formed a gel-like pellet.

The supernatant was removed and the remaining RNA pellet was washed once with 0.5 mL of 75% ethanol and mixed by vortexing. The mixture was centrifuged at

7,500 x g for 5 min at room temperature and the ethanol was decanted. The RNA was then air dried for 10 min, dissolved in 100% deionized formamide and stored at -70ºC to be used in RT-PCR analysis.

Semi-quantitative one step RT-PCR analysis: The extracted total RNA, Superscript III RT/Platinum Taq Mix, Reaction Mix, 5 nM Magnesium Sulphate, GeneAMP PCR System thermal cycler were used according to the manufacturer's instruction. The CB_1 primers, sense 5'-GAT GTC TTG GGA AGA TGA ACA AGC-3' (nt 365-373) and antisense 5'-AGA CGT GTC TGT GGA CAC AGA CAT GG-3' (nt 460-468). The primers were selected from Esposito *et al*. (2002), since they were used for the study of CB_1 expression and the effect of cannabinoid agonist treatment in neuronal glial cells. Alpha actin primer Sense 5'-GAT CAC CAT CGG GAA TGA ACG C-3' (389bp) and Antisense 5'-CTT AGA AGC ATT TGC GGT GGA C-3', selected from Park *et al*. (1997), where they were used as an internal control for cytoskeletonal study in pericytes.

Programming of the thermal cycler was done as follows: cDNA synthesis 1 cycle at 55ºC for 30 min, Denaturation 1 cycle at 94ºC for 2 min, PCR amplification 40 cycles at 94ºC for 15 sec (Denature), 60ºC for 30 sec (Anneal), 68ºC for 60 sec (Extend), Final extension, 1 cycle at 68ºC for 5 min.

The master mix was prepared on ice using 0.2 mL nuclease free, thin walled PCR tubes. Each PCR tube contained the following: 2 x Reaction mix (dNTPs: 200 µM; $MgSO_4$: 1.6 mM) 25µL, Template RNA (200 ng/µL) 1µL, Sense Primer (0.2µM), 1 µL, Antisense Primer (0.2 µM)1 µL, SuperScript III RT/ Platinum Taq mix (5 mM) 2 µL, Autoclaved distilled water; 20 µL was added to make up total volume to 50 µL.

These were mixed together gently and all the components were allowed to settle at the bottom of the amplification tube. The tubes were then centrifuged briefly by pulse centrifugation, over-laid with one drop of mineral oil and placed in a preheated thermal cycler (GeneAMP PCR System) as programmed above. One tube was used as blank and contained only master mix and water.

Analysis of the RT-PCR products: The analysis of the PCR products was carried out using the following method. Agarose gel (2%) was made by dissolving 2.5 g agarose in 112.5 mL of distilled water which was subsequently micro-waved in a conical flask for 5 min. 12.5 mL buffer solution of Tris/Borate/EDTA (TBE) was added and 5 µL of ethidium bromide added for staining the mRNA and mixed thoroughly. The mixture was poured into the gel tray with combs in place and air bubbles pushed to the sides with a pipette. The gel was allowed one hour to set. 5 µL of Blue loading buffer (Sigma, G7654), which contain bromphenol blue (0.25% w/v), xylene cyanole FF (0.25% w/v), sucrose (40% w/v), was added to each of the samples and centrifuged briefly to mix and settle.

A 10 µL DNA ladder (Promega, G2101) and 5 µL Blue loading buffer was used to make-up the DNA marker, and 1 L of gel buffer was made using the TBE in a 1:10 dilution. The gel was placed in an electrophoresis tank with wells at the negative electrode such that RNA will move towards the positive electrode. The gel was then surrounded with gel buffer and the wells completely covered by the buffer. The DNA marker (5 µL), was added to the gel on position one, followed by adding a 10 µL blank sample to the next position, and continuing to add 10 µL of each sample to the remaining wells. The electrophoresis tank was connected to a power supply set at 125 V for 1 h. Photographs of the gels were taken and scanned using the digital densitometer to evaluate and semi-quantify the mRNA of the receptors, and then compared between the different groups.

Statistical analysis: The different parameters measured from the normal, hypoxic and treated experimental groups of B50 neuronal cells were compared using mean and standard deviation (SD). The parameters were assayed in triplicate and repeated twice (n = 6) and the results presented as the mean±SD. The Students't-test was used for testing the level of significance between two groups and a P-value less than 0.05 was considered to be significant using Microsoft Excel® package. For multiple treatment data, One-Way analysis of variance (ANOVA) was used followed by Multiple Range Test post hoc subgroup testing to find the least significant difference (LSD) between the groups.

RESULTS

Morphological changes were observed in B50 cells cultured under hypoxia when compared to cells cultured in normoxia. The B50 cells in hypoxia showed clustered groups of neuronal B50 cells, evidence of degenerating, dying cells and already degenerated and dead neuronal B50 cells. The normal B50 neuronal cells cultured under normal incubator showed normal neuronal morphology (Plate 1 and 2), when compared to B50 cells in hypoxia (Plate 3 and 4).

The effect of hypoxia on the expression of CB_1 receptor gene in B50 cells: The result of CB_1 receptor gene expression in B50 neuronal cells in normal, hypoxic and treated cells was studied using reverse transcription polymerase chain reaction (RT-PCR) (Fig. 1). The results showed that the RT-PCR experiments with the B50 neuronal cells in normal, hypoxic and treated cultures demonstrated positive gene expression of the cannabinoid

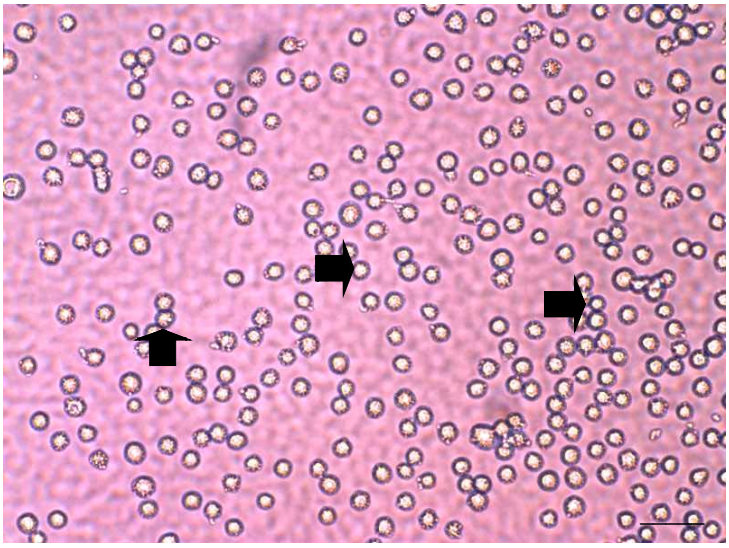


Plate 1: Representative of B50 cells at other with normal B50 cells (arrow) at the point of starting the culture at 21% O_2 and 5% CO_2. B50 cells were observed in three different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar=5mm×40 magnification.

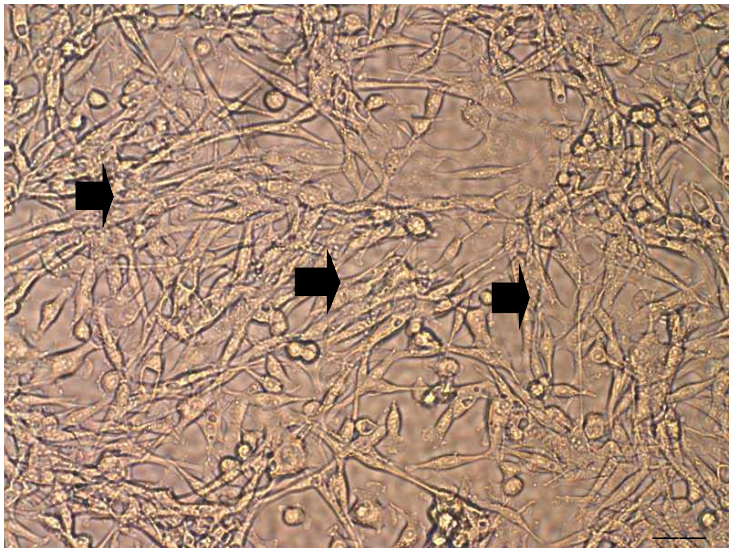
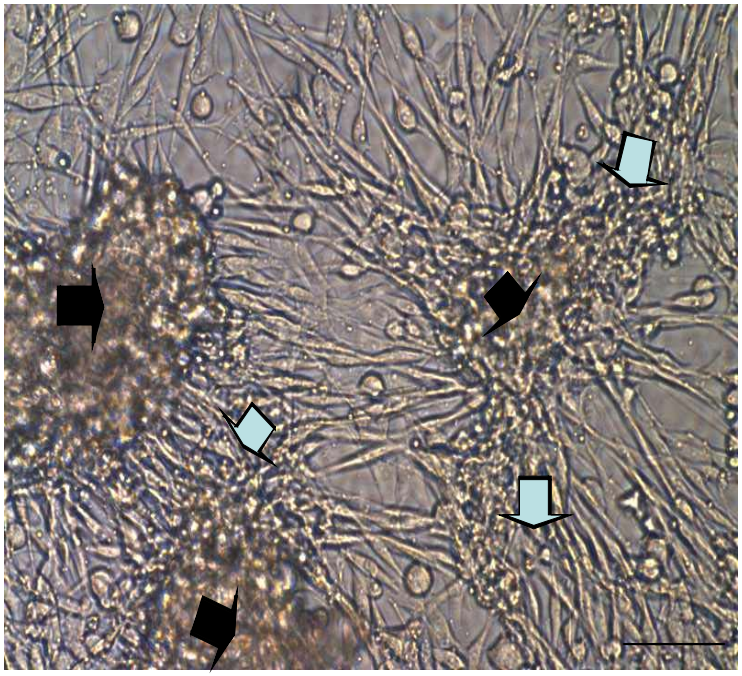


Plate 2: Representation of B50 cells at 48hrs of normal culture (21% O_2 and 5% CO_2) with B50 cells (arrow). B50 cells were observed tn three different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS 100 microscope and microphotographs processed with IBM Image Solution® . Scale bar=5mm×40 magnification

CB_1 receptors. The mRNA levels of CB_1 receptors in hypoxic culture of B50 cells were expressed relative to CB_1 receptors in B50 cells cultured under normal conditions, and these were also compared with mRNA levels of CB_1 receptors in hypoxic B50 cells treated with different receptor agonists (Fig. 2). The results showed that there were no significant difference in the levels of CB_1 receptor gene and mRNA expression between the normal, hypoxic and agonist treated cultured B50 cells.

Semi quantitative RT-PCR of cannabinoid CB_1: CB_1 receptor gene expression products were subjected to semi quantitative analysis using digital densitometric measurements. The result showed a significant decrease ($p<0.05$) in the density of the RT-PCR products of CB_1 in hypoxic cells treated with 100 nM AEA (85%) and 10 nM 2-AG (80%) agonists when compared with normal cultured B50 cells (100%), while the increase of 102, 101, 107, 104 and 104% in untreated hypoxic cells and cells



Palate 3: Representative of hypoxic B50 cells at 96 hrs of culture (5% O_2 and 5% CO_2) with groups of degenerating cells (black arrow) and altered pattern of cells arrangement (blue arrow). Groups of degenerating and altered nerve arrangement were observed in six different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS 100 microscope and microphotographs processed with an IBM Image Solutions ®. Scale bar=5mm×40 magnification

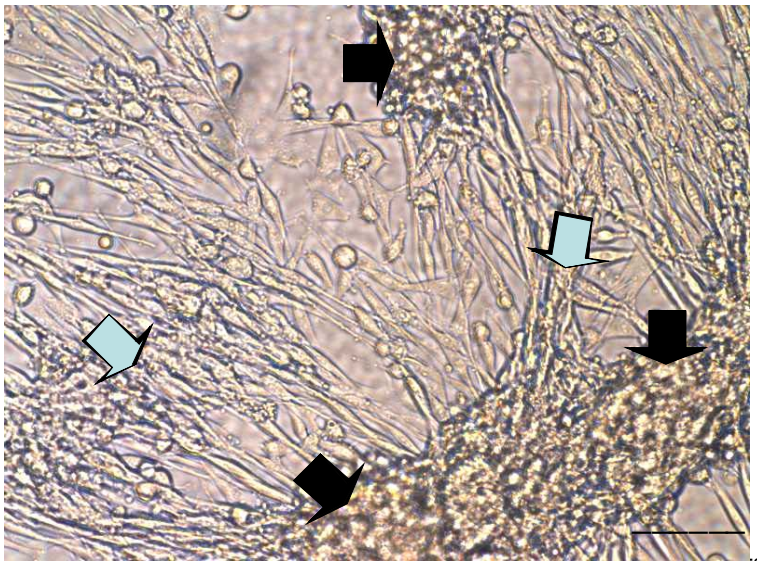


Plate 4: Representative of hypoxic B50 cells 96hrs of culture (5% O_2 and 5% CO_2) with groups of degenerating cells (black arrow) and altered pattern of cell arrangement (blue arrow). Groups of degenerating and altered nerve arrangement were observed in six different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS 100 microscope and microphotographs processed with an IBM Image Solution ®. Scale bar=5mm×40 magnification

treated with 10 nM Win, 50 nM Win, 10 nM AEA and 50 nM AEA, were not significantly different from the control (100%). The decrease in the density of CB_1 receptors with 100 nM Win (97%); 50 nM 2-AG (92%) and 100 nM 2-AG (95%), was not significant when compared with the control. There was no significant change in densitometric

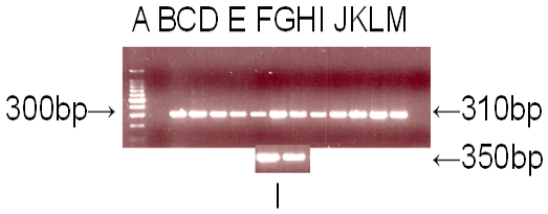


Fig 1: The effect of hypoxia and cannabinoid treatment on CB_1 receptor expression in B50 cells
A: DNA Ladder; B = Normal B50 cells; C = Hypoxic B50 cells;
D-I = Hypoxic treated cells with different cannabinoid agonist;
J-M = Hypoxic cells treated cannabinoid agonist/antagoinst;
I = Alpha actin

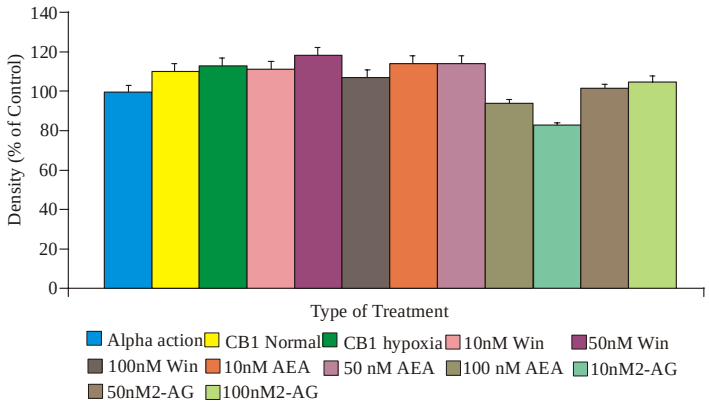


Fig. 2: The effect of hypoxia on the expression density of CB_1 receptor in B50 cells

Table 1: Semi-Quantitative RT-PCR product of CB_1 receptor gene in B50 cells in culture

TreatmentType	Vol.mm^3	Areamm^2	STD ±	Density	Width	Height	Normal (%)	Control (%)
CB_1Norma	09.61	4.17	0.39	571.41	1.40	2.98	100	110.0
Hypoxia	15.75	6.66	0.33	586.22	1.78	3.75	102.59	112.85
10nM Win	12.32	5.24	0.33	577.20	1.58	3.36	101.01	111.12
50nM Win	10.59	4.28	0.35	612.24	1.77	2.40	107.15	117.86
100nM Win	6.35	2.83	0.35	555.37	1.21	2.35	97.19	106.91
10nM AEA	10.65	4.44	0.35	595.31	1.40	3.18	104.18	114.60
50nM AEA	5.91	2.60	0.35	595.31	1.40	3.18	104.18	93.98
100nM AEA	3.91	1.98	0.21	488.19	0.76	2.60	85.44*	83.37
10nM 2-AG	6.99	3.53	0.03	459.07	0.38	1.40	80.34*	101.53
50nM 2-AG	8.64	4.06	0.30	527.40	1.21	3.37	92.30	104.65
100nM 2-AG	8.90	4.06	0.31	543.60	1.21	3.37	95.13	104.65
Alpha actin	10.35	3.22	0.32	519.46	2.02	3.12	90.91	100

measurements of mRNA area, mRNA volume, height and width respectively when compared with the control B50 cells (Table 1).

When the density of the RT-PCR products was normalised to alpha actin (100%), there was no observed difference in the levels of the receptor gene mRNA expression of CB_1 in normal, hypoxic and treated cultured B50 cells. The results showed no significant difference between the normal (CB_1) cultured B50 cells (110%), untreated hypoxic cells (112%) and hypoxic treated cells with 10 nM Win (111%); 50 nM Win (117%); 100 nM Win (106%); 10 nM AEA 114%; 50 nM 2-AG (101%). The CB_1 receptor density showed a non-significant decrease with the cells treated with 100 nM AEA (93%) and 10 nM 2-AG (83%), when compared to those of alpha actin (100%) (Fig. 2).

DISCUSSION

The results from the present work showed the cortical B50 neurons cultured under normoxia, hypoxia and hypoxia treated with cannabinoid agonist and antagonist,

positively showed G protein coupled (CB_1) receptor gene expression using RT-PCR method. The results also demonstrated that there was no appreciable change in the levels of CB_1 receptor mRNA expressed. The effect of cannabinoid receptor agonist treatment on the morphology of B50 cells showed changes in the morphology of the B50 cells cultured in hypoxia when compared to the normal and untreated hypoxic B50 cells. The changes in the morphology could be correlated with changes in viability and LDH leakage from the B50 cells, results not shown here. Some of the results show significant increases in the presence of the antagonist, suggesting that the actions of the agonists may be mediated through both receptor- and non-receptor-mediated pathways in these cells. The mediation through the cannabinoid (CB_1) receptors in this process occurs via the activation of $G\alpha_{i/o}$ proteins. This could explain why the hypoxic and hypoxic treated cells both showed positive expression of the CB_1 receptor genes though some of the cells are dying and degenerating in culture and the effects of hypoxia differentially affected them. Aguado *et al*. (2007) have shown that the CB_1 receptors mediate excitotoxicity-induced neural progenitor proliferation and neurogenesis. It has been shown that the endocannabinoid system exerts an important neuro-modulatory function in different brain tissues and is known to be involved in the regulation of neural cell fate (Galve-Roperh *et al*., 2007). There is the presence of a functional endocannabinoid system in neural progenitor cells that participates in the regulation of cell proliferation and differentiation which play a regulatory role in neurogenesis (Galve-Roperh *et al*., 2007).

The endocannabinoid system has been shown to affect early progenitors and this extends to the regulation of neuronal migration and the attainment of the morphological, physiological and molecular characteristics that occur during terminal neuronal differentiation (Harkany *et al*., 2007). It has also been shown that AEA and Win, with brain-derived neurotrophic factor, induce the migration of GABA-containing interneurons that undergo migration to populate the embryonic cortex (Harkany *et al*., 2007). The activation of the CB_1 receptors in which downstream signalling events such as proliferation and differentiation occur, exert profound effects on neurite outgrowth and synaptogenesis (Harkany *et al*., 2007; Berghuis *et al*., 2004; Berghuis *et al*., 2005). The cannabinoid agonist, HU210 promotes neurite outgrowth in Neuro 2A cells by the $G\alpha_{o/i}$-mediated degradation of Rap-GAPII and the subsequent activation of Rap1 (Jordan *et al*., 2005). These effects could be as a result of the level of G protein Coupled receptor (CB_1) gene expression in B50 cells.

The results of the present study showed some consistency with other studies showing that the different cannabinoid receptor agonists used acted through the G protein coupled receptors (GPCRs) to mediate cellular activities, functions and alterations via the intracellular second messenger pathway. These cellular activities such as proliferation and differentiation were shown to increase in the cells treated with the cannabinoid agonists when compared with the untreated hypoxic B50 cells in culture (Zhuang *et al*., 2001).

The involvement of CB_1 receptors in cannabinoid neuroprotective effects of Win and AEA, was because of the ability of the cannabinoid antagonists to inhibit the protection from the agonists, thus pointing to a $G_{i/o}$-mediated mechanism. This is because exogenous cannabinoids have been shown to protect against neurotoxicity in a number of different cellular, animal and human experimental models (Pryce *et al*., 2003; Davies *et al*., 2002; Wang *et al*., 2007). Zhang *et al*. (2005) have demonstrated that cultured rat hippocampal neurons were protected from excitotoxic insults by pre-treatment with either Δ^9-THC or Win and these compounds were effective in preventing cell death even if administered prior to the neurotoxic exposure. The results presented here showed that the CB_1 mRNA expression thus the CB_1 gene expression were unrelated to the levels of protection exerted by cannabinoid agonists. Thus, the results of CB1 gene expression in B50 neurons are likely the result of more complex interactions within these B50 cells. It is possible to reflect that CB_1 receptors are found in the cerebral cortex especially in the medial frontal cortex. Hence input from the cortex affects gene expression in other neurons (Steiner *et al*., 1999), and a study indicates that cortical activation may preferentially increase gene expression in striato-pallidal neurons (Steiner *et al*., 1999). Therefore, it is conceivable that altered inputs from the cortex, or other brain areas, contribute to changes in gene expression in the striatum in CB_1 mutants (Steiner *et al*., 1999).

Three mechanisms could be attributable to the reactions of the agonists used in this study. The G protein coupled receptors involved could be over activated, desensitized or saturated. Thus at higher concentrations of cannabinoid administration, the receptors could either become over-activated, desensitized or become saturated and hence leading to the higher concentrations of the drugs not resulting in higher response to the drug action. It could also be that at higher concentrations of the drugs, the receptors become highly activated and hence cell death could result, leading to elevated LDH release from the B50 cells in culture. The mechanism involved could also be that the receptor stimulation by higher concentrations of the agonists evoked Ca^{2+} release from the endoplasmic reticular stores followed by Ca^{2+} influx through store-operated Ca^{2+} channels in the plasma membrane (Chang *et al*., 2007). This effect could also be

an example of a specialized functional homologous or heterologous desensitization of the CB_1 receptors in higher concentrations of the agonists which induces interference on the receptor-induced activities. This is because Van-Ham and Oron (2005) have shown that $G\alpha_o$ G-proteins mediate both homologous and heterologous rapid desensitization of responses mediated by G-protein-coupled receptors (Downer *et al*., 2003; Sarne and Mechoulam, 2005).

The cannabinoid agonist-induced stimulation of CB_1 receptors leading to cannabinoid receptor-mediated stimulatory effect on cAMP accumulation in the cells is through the $G\alpha_s$ (Glass and Felder, 1997). The cannabinoid-induced increase in cAMP accumulation in B50 cells when compared to untreated hypoxic cells, results not shown, may lead to the increased down-stream signalling activities such as proliferation and differentiation in cannabinoid-treated cells, through a combination of molecular signalling pathways in B50 cells (Yao *et al*., 2006). Most of the protectant effects of cannabinoids appear to be mediated by the activation of the cannabinoid (CB_1) receptors (Grundy, 2002; Mechoulam *et al*., 2002; Biegon, 2004). These effects were blocked by the cannabinoid receptor antagonists AM251 and AM630, suggesting both cannabinoid receptor- and non-receptor-mediated mechanism. Cannabinoids have been reported to protect neurons from death caused by glutamatergic over-stimulation, ischaemia and oxidative damage (Hansen *et al*., 2002). These effects could have been due to the cannabinoids having multiple effects on the G proteins mediated responses (Slessareva *et al*., 2003). The differences between the effects observed in different models may be related to the cell type or model system used and the differences in the toxic events which have been employed (Chen *et al*., 2005; Drysdale and Platt, 2003; Zhang *et al*., 2005).

In conclusion, the present study shows that the RT-PCR quantification of cannabinoid (CB_1) receptor mRNA can be used to monitor the receptor transcription *in vitro* and direct detection of the effects of receptor signalling during hypoxia in cultured B50 cells. The RT-PCR method has also shown to be good in the study of the effects of cannabinoid agonists in the expression of CB_1 receptors in B50 cells in culture conditions. The results from the present study showed that hypoxia induced morphological changes in B50 cells in hypoxia while the CB_1 RT-PCR mRNA levels showed no appreciable changes in normal, hypoxic and cells treated with cannabinoid agonists. The results show that B50 neuronal cells are susceptible to damage and injurious effects of hypoxia, as are most brain cells and the cannabinoid agonist treatments showed there were no changes in the level of CB_1 receptor gene expression due to hypoxia or agonist treatment in neuronal B50 cells in culture.

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